A STUDY ON EGG DROP SYNDROME 76 (EDS76)

IN KHARTOUM STATE

By

Rihab Muhammad Dafallah Hamad Elneel

B.V. Sc. University of Nyala (2001)

Supervisor

Dr. Suliman Mohammed El Hassan

Co-Supervisor

Dr. Abed El Gadir Ballal Mohammed

A thesis submitted to the University Of Khartoum in partial fulfillment of the requirement for the degree of Master of Science,

Department Of Microbiology

Faculty of Veterinary Medicine University of Khartoum

May 2006

DEDICATION

To my dear mother

To my dear Father

To my brothers and Sister

To all whom I love

ACKNOWLEDGMENT

First and fore most, my heart felt thanks to the merciful Allah for giving me the power and health to complete this work. I would like to express my special appreciation to my supervisor Dr. Suliman, Mohammed Elhassan for his help, suggestion, advice and encouragement throughout this study.

My thanks go also to my cosupervisor Dr. Abdel Gadir Ballal for his help and advice.

My deepest thanks to Dr. Sobhi Mohammed Kheir who help me kindly. My thank to all the staff of Department of poultry diseases, of Central Veterinary Research laboratory in Soba.

Special thanks to my friend Wegidan Hassan Ali for help during the laboratory work. Great thanks are also extended to Dr. A wad Mahjoob and Prof. Abdel Raheem Gameel for their assistant.

I am greatly indebted to my friend Omsalama Khalaf Allah and Etidal Mustafa for their kindness and also to all my colleagues for their support and assistance.

Finally, I would like to express my sincere gratitude to my family and all my friend who were not mentioned and helped to make this work possible.

CONTENT

LIST OF TABLES

LIST OF FIGURES

SUMMARY

The present work was carried out to study Egg drop syndrome in Khartoum State. The aims of the work are to collect field observations on the disease, to carryout serological survey to detect antibodies against the virus and to isolate and identify the virus.

An outbreak of the disease occurred in different breeders in Khartoum State during the period of the study. Owners of many farms reported to the Soba CVRL. The age of infected birds were between 28-30weeks. The clinical signs were sudden drop at peak of egg production (the drop was 30%-45%), loss of colour of pigmented eggs, thin shelled eggs or soft shelled. The thin shelled eggs often have a rough sand-paper like texture or granular roughening of the shell at one end. The birds looked quite healthy.

Trial was made to isolate the virus by inoculating chicken eggs, three passages were made in allantoic fluid in 10 days old chicken embryo. Forty five samples collected from 15 cloacal swabs, 15 tissue samples (Oviduct-ovaries-pouch shell) and 15 (intestine-oesophagus) samples. However, no virus was isolated.

Serological examinations to detect antibodies against EDS 76 virus in some laying hens sera, and 31.7% positive sera were detected by (H1) test and 14.2% positive sera by ELISA technique.

موجـز البحث

أجريت هـذه الدراسـة على ظاهرة نقص أنتاجية البيض في قمـة الإنتـاج. وكان الهدف منها الملاحظات الحقلية للمرض ، المسح المصلي للكشف عن وجود الاجسام المضادة وعزل الفيروس والتعرف عليه.

لقد ظهر المرض في عدة مزارع في منطقة الخرطوم بحري والخرطوم خلال فترة الدراسة حيث قام أصحاب المزارع بتبليغ معمل الأبحاث البيطرية بسوبا. عمر الدجاج المصاب حوالي 30 أسبوع والأعراض تشمل النقص المفاجئ في الانتاج (وتقدر بحوالي 30-45%) . وقشرة البيض الرفيعه والهشه ولم يلاحظ عـدم وجود قشرة وتترسب أملاح الكالسيوم فوق القشرة لتعطي منظر الورقه الرملية – وتكون هنالك خطوط متعرجه بأحدى الجوانب – البيض لونه باهت وصغير في الحجم يلاحظ أن الدجاج المصاب يكون طبيعي جداً.

أجريت محاولة لعزل الفيروس بحقن أجنة الدجاج وجمعت العينات لزراعة الفيروس من عدة مزارع تظهر عليها أعراض المرض ، حوالي 45 عينه -15 عينه من مسحة المجمع 15 عينه من انسجة (قناة المبيض– البويضات– غدة باوش) 15 عينة من أنسجة (الأمعاء – المرئ). تم الحقن في الكيس المشيمي لأجنة الدجاج عمر عشرة يوم وقد تم التمرير ثلاث مرات.

جمعت الف عينة سيرم وأجريت الاختبارات السيرولويجيه للكشف عن وجـود الاجسـام المضادة لفيروس نقصان إنتاجية البيض وكانت 31.7% من الأمصال موجبه

لاختبار تثبيط التلازن لخلايا الدم الحمراء بالدجاج بينما كانت 14.2% موجبه لاختبار الاليزا وقد أكدت هذه الدراسة الدراسات السابقة التي قام بها بلال وأخرون عام 1994 وتأكد من نتائج هذه الدراسة والدراسة السابقة لتشخيص المرض معملياً في ولاية الخرطوم لعدم ممارسة التطعيم ضد المرض في السودان.

INTRODUCTION

Egg drop syndrome 76 (EDS 76) is a serious disease of chicken it was first described in the Nether Lands in 1976 (Van Eck *et al*., 1976). The disease is characterized by a reduction in a marketable eggs (up to 50%), eggs produced will be depigmented, soft shelled or shell less, leading to a drop in the number of recoverable eggs and therefore an apparent Egg drop syndrome. Rearing birds are unaffected and the problem appears mostly at peak of production. An adenovirus was identified as a causative agent of the disease (Baxendale 1978, Firth *et al*., 1981).

Ballal and Kheir (1994) investigated the presence of antibodies to EDS 76 in the sera of fowl flocks in Khartoum State. They obtained positive result which indicated the presence of circulating viruses.

This research work aims:

To study the current situation of the disease in Khartoum State by:

1. Detection of EDS 76 antibodies in chicken's sera by means of HI test and ELISA technique.
2. Isolation of the virus using Chicken embryo inoculation.

CHAPTER ONE

1. LITERATURE REVIEW

1.1 The disease EDS 76:

Since the initial description by Van Eck *et al.* (1976), EDS 76 has become a major cause of egg loss throughout the world. It is caused by an avian adenovirus. The disease was introduced from duck into chickens through a contaminated vaccine. (Baxendale, 1978, Firth *et al.,* 1981).

EDS 76 is characterized by otherwise healthy birds producing thin-shelled or shell less egg. Once established in a breeding organization the disease condition is more often seen as a failure to achieve production target and egg shell. Changes are less apparent, although still presents (Van Eck *et al*.,1976). Since its initial recognition, it has become apparent, that sporadic outbreaks of EDS 76 occur as a result of fowl becoming infected through direct or indirect contact with infected wild or domestic water fowl.

1.1 Historical back ground:

The first condition of laying hens was described by Dutch workers in 1976 (Van Eck *et al*., 1976). It was first recognized in Northern Ireland in 1977, when hemagglutinating adeno viruses were isolated (McFerran *et al*., 1977). The virus was introduced into Northern Ireland by birds coming through quarantine in 1974. It appeared that the virus was transvertically transmitted and horizontal transmition between flocks was not a feature.

The virus often remained latent until birds were approaching peak of egg production. Because of absence of antibody to the virus in chickens prior to 1974 and failure of the virus to grow in a mammalian cells as well as its poor growth in turkey cells and optimal growth in duck cells, it was suggested that this was probably a duck adeno virus. This suggestion was quickly confirmed by isolation of EDS 76 virus from normal duck and, demonstration of antibodies in many duck flocks (Baxendale, 1978).

1-1. Egg drop syndrome 76 in the Sudan:

EDS 76 is one of the major diseases throughout the world. Little studies have been done on the disease in the Sudan. EDS 76 was diagnosed in Sudan for the first time in 1994. However, Ballal and Kheir (1994), using HI test, detected antibodies to EDS 76 virus and this indicated pervious adeno virus infection since there is no vaccination program against avian adeno virus EDS 76 adopted in Sudan.

1.4. Avian adenovirus:

Avian adenoviruses were recognized as a cause or contributing cause of a number of diseases of chickens, including respiratory disease, enteritis, inclusion body hepatitis, egg drop syndrome, atrophy of the bursa of fabricius and hemorrhagic- aplastic syndrome (Fenner *et al.*, 1987). The EDS 76 virus belong to DNA viruses, nonenvelope virus that replicates in the nucleus. Avian adenoviruses are divided into three groups. The first group includes the avian group or conventional adenoviruses, which share a common antigen. The second group consists of the viruses of hemorrhagic enteritis, marble spleen and the splenomegaly virus of chicken. The third group contains the viruses isolated from chickens with EDS76 (Zsack and Kiary, 1984).

1.5. Transmission and spread of EDS 76:

It is possible to divide EDS 76 outbreaks into three forms.

1.5.1. The classical form:

The first type, the classical form where primary breeders were infected, the main method of spread was vertically through the embryonated eggs (McFerran *et al*., 1978b, Murphy *et al*., 1999). Although the number of infected embryos is probably low with this type (Baxendale *et al*., 1980, Darbyshire and Peters., 1980), spread is very efficient. In many cases chicken infected *in ovo* do not excrete virus or develop HI antibody until the flock has achieved between 50% and peak egg production. At this stage the virus is unmasked and excreted, resulting in an apparently rapid spread of virus due to multiple foci of infection probably arising from the classical form, virus has become established in some areas in commercial egg-laying flocks.

1.5.2. The endemic form:

The second type is the endemic form an often associated with a common egg-packing station. Both normal and abnormal-shell eggs laid during the period of virus growth in the pouch shell gland contain virus, both on their exterior and interior (Murphy *et al.,* 1999).

This leads to contamination of egg trays, dropping also contain virus, but this excretion is intermittent and often of low titer (Cook and Derbyshire, 1980), and in the adult bird may result from contamination of the feces by oviduct exudate (Jordan, 1990, Murphy *et al*., 1999).

Apart from direct spread between birds, there is evidence that spread can occur when birds are transported in adequately cleaned trucks or when unused food has been taken from one site to an other. There is also evidence that needles or blades used for vaccination or bleeding of viremic birds, if not properly sterilized, can transmit infection. Lateral spread is slow and intermittent, taking up to 11 weeks to spread through a cage house, in one case, spread to an adjoining pen was prevented by a wire fence (Calnek *et al*., 1991).

Spread between birds on litter is usually faster (Van Eck *et al.,* 1976, McFerran *et al.,* 1977, Cook and Derbyshire, 1980, 1981).

1.5.3. Wild birds form

Spread from both domestic or wild ducks, geese and possibly other wild birds to hens through drinking water contaminated by droppings appears to give rise to a third type of outbreak. This type is very important in some areas.

1.6. Classification of EDS 76 virus:

EDS 76 virus is classified as an adenovirus on the basis of its morphology, replication and chemical composition. The adenovirus constitutes the adenovirus family of viruses, which is divided in two genera, Mast adenovirus and Aviadenovirus, whereas the Aviadeno viruses genus is limited to viruses of birds, the Mast adenovirus includes human, simion, murine, bovine, equine, ovine, porcine, canine and opossum viruses (Knip *et al.,* 2001).

A third genus Ata adenovirus was proposed, but had not been accepted by the international committee on taxony of viruses (Murphy *et al.,* 1999). The various serotypes are classified into six subgroups based on their ability to agglutinate red blood cells. The central shaft of the viral fiber protein is responsible for binding to erythrocytes and the hemagglutination reaction of an adenovirus is inhabited by specific antisera to viruses of different types (Knip *et al*., 2001).

A variety of additional classification have been explored including subgrouping based on ancogenicity in rodents, relatedness of tumor antigens, electrophoretic mobility of virion protein, or genome homologies but classification based on haemagglutination is reasonable standard (Knip *et al*., 2001).

1.7. Classification of strain:

Avian adenoviruses have been classified by their serologic relationships, growth in cell culture and nucleic acid characteristics. A number of workers have compared the strain by neutralization tests (Mc Ferran, 1997). There is agreement over serotypes (or species), but some disagreement over serotype designation. Twelve fowl serotypes have been recognized, but there are undoubted by more that have been isolated but not yet classified (Mc Ferran, 1997).

After the general structuring of the family had been redone on the basis of molecular characteristics of the viruses, the bases for the immunologic relationship, among the viruses became clear. The merits of the serological structuring of the family were based more on the relative dominance of certain epitopes in particular serological tests than on their location in the virion (Murphy *et al*., 1999).

1.8. Properties of the virus:

Adenoviruses are Icosahedral particles, measuring 70 to 100 nm In diameter. The viral particles (virions) contains DNA (13% of mass) - protein (87% of mass)- non membrane lipid and trace amount of carbohydrates because the virion fiber protein is modified by addition of glucose-amine (Knipe *et al.,* 2001).

The virion consists of a protein shell surrounding a DNA containing core. The protein (capsid) is composed of 252 subunits (capsomeres), of which 24 are hexon and 12 are pentons. Penton and hexon subunits are surrounded by five and six neighbors, respectively. Each penton contains a base which forms part of the surface of the capsid, and a projecting fiber whose length varies among different serotypes (Knipe et al., 2001).

1.9. Replication of the virus:

Viral DNA replication begins between 5 to 8 hours after infection and continues until the host cell dies (Knipe et al., 2001).

Adenovirus DNA replication takes place in two stages-first, synthesis is initiated at terminus fashion to the other end of the genome only one of the two DNA strands serves as a template for the synthesis, thus the product of the replication are duplex consisting of a daughter and parental strand plus a displaced single strand of DNA. In the second stage of the replication process a complement to the displaced single strand is synthesized. The single stranded template circularizes through annealing of its self complementary termini, and the resulting duplex was the same structure as termini of the duplex viral. This structure allows it to be recognized by the some initiation machinery that operates in the first stage of replication and complementary strand synthesis generates a second completed duplex consisting of one parental and daughter strand.

Adenovirus was the first DNA template to be replicated in *vitro* (Knipe *et al*., 2001).

1.10. Pathogenicity:

The chicken adenovirus is ubiquitous in fowl as demonstrated by many antibody surveys. In addition to infected chicken, fowl adenovirus serotypes have been recovered from turkeys, pigeon, budgeri, gars and duck (MC Ferran, 1997). Although adenovirus can be isolated from day 1 onward, viruses are normally excreted from week 3 onword. In broiler, peak excretion occurred between 4 and 6 weeks (McFerran, 1997). Vertical transmission is very important. Adenovirus is transmitted through the embryonated egg (McFerran, 1997).

Horizontal spread is also important, virus is present in feces, tracheal and nasal mucosa. Virus could be transmitted in all excretions but highest titers are found in the feces. Horizontal spread appears to be mainly by direct contact but also by aerial spread over short distance with a slow spread taking weeks. Aerial spread between farms does not appear to be important but spread by fomites, personal and transport can be very important (Mc Ferran, 1997).

Many studies showed the route of inoculation has been extremely important, many isolates have failed to cause disease when given by parental injection; other workers suggested that adenovirus pathogens require some other agents to allow them to cause disease. Age of chicken is also important. In one day chicken, injection may be possible to cause mortality, but not in 10 day old birds.

Infectious bursal disease virus enhances pathogenicity of avian adenovirus (Mc Ferrran, 1997).

1.11. Clinical signs:

The first sign is a loss of color in pigmented eggs. This is quickly followed by production of thin-shelled, soft-shelled, or shell less eggs. The thin-shelled eggs often have a rough sand paper like texure or a granular roughening of the shell at one end. If obviously affected eggs are descarded, there is no effect on the fertility or hatchability and no long term effect on egg quality. If birds are infected in late production, forced molting of the flock will restore egg production to normal (Calnek *et al.*, 1991). Small eggs have been described in natural outbreaks (Mc Ferran *et al*., 1978, Yomaguchi *et al*., 1981), but no effect on egg size was found in experimental infectious (Mc Craken, 1978). Watery albumen has been described (Van Eck *et al*., 1976, Meulemans *et al*., 1979a). Although no effect on albumen has been seen by other workers (Mc Craken an Mc Ferran *et al*., 1978, Darbyshire and Peters, 1980, Yamauchi *et al*., 1981b). However, age of infection may be important; Cook and Darbyshire (1981) found that birds infected at 1 day of age subsequently laid apparently normal eggs except for impaired quality of albumen and smaller size.

If some birds have acquired antibody before the latent virus is unmasked, an apparently different clinical syndrome is seen. There is failure to achieve predicted egg production, and onset of lay may be delayed. If a careful examination is made, it can usually be established that there is a series of small clinical episodes of classical EDS 76. Presumably, birds with antibody slow down spread of virus. A similar picture is often seen in birds in cage units, where spread can be slow and EDS 76 not suspected, the affected birds remain otherwise healthy. Although inappetence and dullness have been described in some affected flock, these are not consistent findings (Calnek *et al*., 1997). The transient diarrhea described by some authors is probably due to the exudates from the oviduct (Taniguchi *et al*., 1981).

Most workers agree that EDS 76 virus does not cause disease in growing chickens in the field. Oral infection of susceptible day-old chickens resulted in increased mortality in first week of life (Cook and Darbyshire, 1981), but there was no increase in mortality in many flocks of chicken produced by infected parent flocks (Van Eck *et al*., 1976, Mc Ferran, 1997).

1.12. Gross lesions:

Following experimental infection, edema of the uterine folds and presence of exudates in the pouch shell gland commonly occurs within 9 - 14 days (Firth *et al*, 1981. Bragg *et al*., 1991). There is also mild splenomegaly, flaccid ovules and eggs in various stages of formation in the abdominal cavity.

1.13. Diagnosis:

1.13.1. Clinical signs and gross lesions:

The diagnosis is achieved by clinical signs and gross post mortem lesions described above. The diagnosis is confirmed by isolation and identification of the causative virus.

1.13.2. Isolation and identification:

The positive indicators system is either embryonated duck or goose eggs from a flock free of EDS 76 virus infection or duck and goose cell culture. Embryonating chicken eggs are not suitable. If these are not available, chicken cells should be used. Chicken embryo liver cells are more sensitive than chicken Kidney cells and chicken embryo fibroblasts are insensitive (Adair *et al*., 1979a. Bragg *et al*., 1991).

Extensive passage is partly due to poor growth of these virus on primary isolation in chicks cells and partly because virus excretion from chickens is intermittent and often of low titer (Calnek *et al*., 1991).

It is not sufficient to rely on embryo death or cytopathic effects with EDS 76 viruses. The allantoic checked after each passage for presence of hemagglutinins for avian erythrocytes (0.8% chick erythrocyte suspension) is suitable. Alternatively immunoflurescence using a labeled antiserum to EDS 76 virus can be used to detect growth in cells (Calnek *et al*., 1991).

When using duck cells a minimum of 2 passages are required and with chicken cells 2 -5 passages are necessary (CaLnek *et al*., 1991).

1.13.3. Serological investigation:

1.13.3.1. Hemagglutination inhibition test (HI):

The HI test is the one of choice for studing this virus. Antigen can be prepared in either embryonated duck eggs or cell culture. Higher HA titers are obtained if duck eggs are used or from chick embryo liver cell cultures. A suitable HI test uses 4HA units of antigen, and initial 1:4 serum dilution and 0.8% chicken RBCs. The virus will agglutinate erythrocytes from chicken, geese, turkeys and ducks but not mammal. There is no hemolysin. If non specific haemagglutinins are present in serum they can be removed by adsorption with a 10% erythrocyte suspension or homologous erthrocytes can be used (David *et al.,* 1998).

1.13.3.2. Serum Neutralization test:

Serum neutralization test using 100Tcid Ihr at 37°C reaction time and duck or chicken cell cultures as the indicator system is sensitive and specific. When using chick cell cultures the end point are read by presence of haemagglutinins in the supernatant fluid rather than by cytopathology. The SN test is really only required to confirm an unusual HI result, as in an eradiation program or detection of HI antibody in a new species (David *et al*., 1998).

1.13.3.3. Fluorescent antibody test:

The indirect (FA) test is at least as sensitive as the HI test. (David *et al*., 1998).

1.13.3.4. Double immune diffusion test.:

The double immune diffusion (DID) test has also been used but it is probably less sensitive than the HI test (David *et al*., 1998).

1.13.3.5. Agar gel diffusion test:

The test has also been used but it is probably less sensitive than HI test (Darbyshire and Peters, 1980).

1.13.3.6. Enzyme-linked immune sorbant assay:

The enzyme-Linked immune sorbant assay (ELISA) technique has been used to detect group–specific antibody and type-specific antibody to the conventional adenoviruses. ELISA is also very sensitive antibody to EDS76 virus. However, ELISA can give false positives in birds with high titers of group antibody to conventional adenoviruses, and therefore, the HI remains the test of choice for diagnosis of EDS 76 (David *et al*., 1998; Raj *et al*., 2004).

Many flocks containing birds infected in ovary do not show antibody during the growing period, and it’s only apparent immediately following egg changes. Therefore, even a negative serologic test of all birds in a flock at, say, 20 weeks of age gives no gurantea of freedom from infection.

If a major fall in production is found, most birds tested will have antibody. However, if failure to achieve predicted levels of production because of EDS 76 virus is the problem, the probable cause is a series of small EDS 76 episodes, which reflect its poor lateral spreading ability. Under these circumstances, care must be taken to select samples through out the house, otherwise false-negative results will be obtained (Calnek *et al*., 1991).

1.14. Treatment:

There is no successful treatment. Various treatments have been tried (giving vitamins, increasing calcium or protein in the ration), in controlled trials but no effect could be demonstrated (Calnek *et al*., 1991).

1.15. Prevention and control:

1.15.1. Management procedures:

Since classical EDS76 is primary spread by vertical transmission through the egg, birds should be derived from uninfected flocks. Endemic EDS is often associated with a common egg- packing station where contaminated egg trays can be a major factor in spread. Virus is also present in dropping and lateral spread is possible because the viruses is resistant. There is circumstantial evidence for spread by personal and transport and therefore sensible hygienic precautions are required (Calnek *et al*., 1997).

Infected birds have aviremia, thus it is important bleeding needles for inoculating vaccines and other equipment should be sterilized between uses.

If there are infected and non infected breeding flocks with the same organization, separate hatcheries, staff, and transport should be used. If this is not possible, separate setters and hatchers should be used, and hatches should be on different days of the week (Calnek *et al.,*1997).

The minimum possible procedure (and this is not recommended) is to use separate hatchers and to sex, vaccinate, dispatch the clean stock before doing anything with potentially infected chicks. It is especially important to keep basic or, grandparent breeding stock of another breed, these eggs should never be incubated in the same hatchery. In certain areas of the world, especially where birds have water derived from dams, lakes or rivers EDS infection has been common. These outbreaks have been controlled either by using water from wells or by chlorination of the water. In units where ducks or geese are kept, they should be carefully segregated from chickens. If possible all housing should be made that wild bird proof. It is established that wild ducks and geese are often infected, but is not known how wide spread infection is in other avian species (Calnek *et al*., 1997).

1.15.2. Immunization:

An oil adjuvant inactivated vaccine is widely used and birds are vaccinated between 14 and 16 weeks of age. If uninfected birds are vaccinated, HI titers of log 8 -9 can be expected; if the flock has been exposed previously to EDS 76 virus, titers of $\log_2$ 12 -14 can be found (Baxendale *et al*., 1980, Cook and Darbyshire, 1981). However, experience in the field suggests that vaccine titers may not always be as high or uniform. The vaccine gives good protection against clinical disease and reduces amount of virus excreted (Calenk *et al.,* 1997).

1.16. Eradiation:

EDS 76 was successfully eradicated from a breeding or organization in Northern Ireland. The method was based on a number of postulation. Chickens produced from infected eggs may be latently infected and fail to develop antibody, the virus will become unmasked and excreted around peak of egg production and antibody will develop which will prevent or reduce further excretion and lateral spread is poor (Calnek *et al.,* 1997).

The eradiation program was based on the elitc and grand parent flocks aged 40 weeks or more. At this stage, these flocks had HI antibody. The chicks hatched from these eggs were divided into small groups of about 100 (separated by nitting wire). They were HI tested at 10 -25% level at about 6 week intervals. If one or two reactors were found they were removed, 100% of the pen and 100% of adjoining pens were then tested twice at weekly intervals. If a number of reactors were found, reactors kept appearing in a pens the whole pen was removed and the in contact pens were tested (Calnek *et al*., 1997).

At 40 weeks test was carried out on all birds and eggs were collected for the next generation. This program was successful; subsequently, the grand parent and parent flocks have been free of infection other precautions taken were that foot wear was disinfected between pens and while a number of bleeding needles were used in each pen, they were placed in 70% alcohol between use (Calnek *et al*., 1997).

CHAPTER TWO

2. MATERIAL AND METHOD

2.1 Collection of samples:

Thousand samples consisting of blood serum were collected from different breeds (hisex -Bovans and local chickens) in Khartoum state. Forty five samples consisting of 15 tissue samples (oviduct-ovaries-pouch shell gland) 15 samples consisting intestine–oesophagus and 15 cloacal swabs were also collected.

2.1.1 Blood

The blood samples were collected aseptically from the jugular or wing vein of chicken or at slaughtering, by 3ml sterile disposable syringes. Then transferred to sterile bijou bottle and left to clot in a slope position at room temperatures. The clot was then separated from the edges of the bijou bottle by sterile fine wire. Sera were then collected, clarified by centrifugation at 2000 rpm for 5 minutes and stored at - 20ºC until examined by HI and Elisa tests.

2.1.2 Organs:

Oviducts, ovaries, pouch shell glands intestine and oesophagus were placed into sterile labeled bottles and stored at-20ºC until inoculated into embryonating chickens.

2.1.3 Swabs:

Cloacal swabs were aseptically collected and placed into sterile labeled bottles and stored at -20ºC until inoculated into embryonating chickens.

2.2 Reagents:

2.2.1 Normal saline:

This was prepared by dissolving 8.5 grams of sodium chloride in 1000ml distilled water and sterilized by autoclaving at 121ºC for 15 minutes. (Barrow and Feltham, 1993).

2.2.2 Phosphate buffer saline (PBS) pH. 7-4:

This was prepared as follow:

Solution A:

Na Cl	8.00g
KCl	0.20g
$N_a H PO_4$	1.15 g
$K H_2 PO4$	0.20g
Distilled water	800 ml

Solution B:

$Ca Cl_2$	0.10 g
Distilled water	100 ml

Each solution was prepared in a separate container and autoclaved at 121 ºC for 15 minutes. After cooling, solution B and C were added to solution A and stored at 4ºC (Barrow and Feltham,1993).

2.2.3 Alsever's solution:

This was prepared as follow:

Dextrose	20.5g
Na Cl	4.2g
Sodium citrate	8.g
Citric acid	0.55g

The mixture was completed to one liter with distilled water and autoclaved at 115ºC for 10 minutes.

2.2.4. Antibiotic solution:

Five ml of benzyl penicillin 10.000 iu/ml, streptomycin sulphate 10.000 iμ/ml and fungizone 250mg/ml [Bio-Whittaker] were dissolved in 95ml PBS-(OIE manual, 1992).

2.3. Sterilization:

2.3.1. Glass ware and instruments:

Pipettes, Petri dishes, test tubes, scissors, forceps, scalpes, pestle and mortars were sterilized in the hot air oven at 160ºC for two hours.

2.3.2. Solutions:

Buffer solutions, saline and rubber caps were sterilized by autoclaving at 121ºC for 15 minutes.

2.4 Antisera:

Known positive and negative (EDS 76) sera (Intervet International), were kindly obtained from the Department of Viral Vaccine Production of the Central Veterinary Research Laboratory (CVRL).

2.5 Antigens:

EDS 76 (HA) antigen (Intervet International), were kindly obtained from the Department of Viral Vaccine Production of the Central Veterinary Research Laboratory (CVRL).

2.6 Serological test:

For the detection of EDS 76 antibodies, two serological tests (HI and ELISA) were used.

2.6.1 Hemagglutination inhibition (HI) test:

2.6.1.1 Preparation of 0.8% chicken red blood cell:

Whole blood was collected from the jugular vein of healthy chickens in Alsever's solution. The blood was washed three times by centrifugation at 1500 rpm for 10 minutes each time. The supernatant fluid was discarded and the red cells were resuspended in sterile normal saline in 5 times their original volume. The suspended red blood cells, were then centrifuged at 1500 rpm for 10 minutes and the supernatant fluid was discarded. Finally 0.8% suspension of chicken red blood cells were prepared in Normal Saline (Grist, 1979).

2.6.1.2 Procedure:

The test was conducted as described by Alexander *et al.* (1983). The antigen prepared was titrated by hemagglutination test as follows.

A) Duplicate of two –fold serial dilution of the antigen was made in v shape micro titer plates (Titer teck, Flow laboratories, U.S.A). Each well in row A and B was filled with 0.050ml of diluents (Normal Saline). In the first well of row A, 0.050ml of the antigen was added and mixed well with fine pipette then 0.050ml of the mixture in the first well was transferred, to the next well. Then mixed and transferred to next well and this was repeated to the last well to prepare to the last well to prepare double fold serial dilution of the antigen. After preparation of the two–fold serial dilutions, 0.050.ml of 0.8% R.B.C suspension was added to all wells and the contents were lightly mixed with a microplate shaker (Cook microtitre system, Denle Tech Ltd, England) and incubated at 4ºC for 45 minutes. The highest dilution at which complete agglutination of the red blood cells occurred was considered as the end point. The titer of the antigen was taken as reciprocal of the end point dilution. The antigen was diluted in N.S to contain four haem agglutination Units (4-HAU) in 0.025ml for use in the HI test (McFerran *et al.*, 1977).

B)Hemagglutination Inhibition (HI) test:-

Each micro titre plate was used for testing 6 sera, a known positive serum and a known negative serum. A quantity of 0.025ml of Normal Saline was added to each well 1-12 in the micro titer plate. To the first well in each row (A to F) 0.025ml of the test serum was added. To the first well in row G, 0.025ml of a known positive serum was added and to the first in well row H, 0.025ml of a known negative serum was added. Then two-fold serial dilutions of the serum were made from well No. 1 to 11 and 0.025ml of the mixture was discarded in each row from well No. 12. To each well in rows from A to H, 0.025ml of the antigen dilution containing 4HAU was added. The serum antigen mixtures were shaken using microplate shaker (Cook Micro titre system, Derely Tech LTD, England), for one minute and then incubated at room temperature for 25-30 minutes. To each well in rows 1-12, 0.025ml (0.8% RBC) was added and incubated at room temperature for 45-60minutes. The test was read when the positive serum reached its titre or when red blood cells in the control were sedimented in button like pattern at the botton of the wells (McFerran *et al.,* 1977).

2.6.2. Enzyme – linked immuno sorbant assay (ELISA):

For the detection of specific antibodies against the EDS virus in avian serum. CIVTEST Avi EDS (Laboratories HIPRA, S.A…..Spain) which is an indirect Elisa for the detection of specific against the EDS virus in serum was used in this study.

2.6.2.1 Principle of the test:

The specific antigen (EDS 76) is coated on 96 well plates. Up on incubation of the sample in the test well, antibody specific to EDSvirus binds with the coated antigen and remains in the well after washing off the unbound materials then a conjugate is added that binds to any attached chicken antibody after that, unbound conjugate is washed away and enzyme substrate is added. The colour appearing in each well is proportional to the amount of chicken antibody specific to EDS virus antigen present in the diluted sample.

2.6.2.2 Instruments

Incubator, precision single and /or multichannel pipettes with disposable pipette tips, tubes or dilution plate for diluting samples, 96-well plate, reader with a 405nm filter and a plate washing device.

2.6.2.3 Kit compositions:-

Products

1- Microplate with 96 wells (in eight-well strips) coated with the specific EDS virus antigen.
2- Washing solution (10x) containing preservative.
3- Sample diluents solution (10x) containing green dye.
4- Conjugate solution: Horse radish peroxidase labeled rabbit anti chicken Igy solution ready to use containing stabilisers, preservative and red dye.
5- Substrate solution: 2, 2 Azino diethyl benzothiazoline sulfonic acid (ABTS) solution ready to use.
6- Stop solution: Oxalic acid solution ready to use.
7- Positive control: Positive control serum prediluted and ready to use containing preservative and yellow dye.
8- Negative control; Negative control serum prediluted and ready to use containing preservative and blue dye.

2.6.2.4 The procedure:

The test was performed according to manufacture instructions as follows.

1- The reagent was allowed to come to room temperature adequate mixing by swirling or inversion was ensured.

2- Sample and control location was recorded on a12x8 template sheet. The positive and negative control was run in duplicate.

3- The adhesive cover was removed from the plate and 50ml of the undiluted controls was added and 50ml of the 1/500 diluted samples was retained at 4°C until successful results were confirmed.

4- The plate was covered with the adhesive cover and incubated at 37°C for 30 minutes.

5- The adhesive cover was removed. The plate was washed three times with reconstituted washingsolution (300Lperwell).Inverted and firmed tap dry on absorbent paper.

6- Conjugate solution 50ml were added to each well.

7- The plate was covered with the adhesive cover and incubated at 37°C for 30 minutes.

8- The adhesive cover was removed.The plates was washed three times with reconstituted washing solution (300ml perwell). The plate was tap dried.

9- Substrate solution 50ml was added to each well.

10. The plate was covered with the adhesive cover and incubated in the dark at 37°C for 30 minutes.

11. Remove the adhesive cover and add 50ml of stop solution to each well. The well contents were mixed.

12. The under-surface of the plate was wiped free of dust with a soft tissue.

13. The plate was read using microtiter plate reader at 405nm having first blanked on air. The results were recorded.

Interpretation of the results:

For the interpretation of results, sample value related to positive value, an S/P ratio was calculated.

$$S/P = \frac{\text{Sample } OD_{405} - \text{Mean } OD_{405} \text{ Negative}_{405} \text{ control}}{\text{Mean } OD_{405} \text{ Positive control} - \text{Mean } OD_{405} \text{ Negative control}}$$

Titre calculation: $\text{Log titre} = 1.207 \times \log_{10} \frac{S}{P} + 3.3736$

Titre = Anti log of $\log_{10}$ titre

The results were interprelated as follow:

S/Pratio	EDS Titre	EDS Antibody status
Less than or equal to0.298	0-548	Negative
Greater than 0.299 and less than458	549 – 921	suspected
Greater than 0.458	922 or greater	positive

2.7 Isolation and identification of virus

2.7.1 Preparation of inoculums:

(a) Swabs were rotated for one minute into normal saline and the fluid was centrifuged at 1000 rpm for 5 minutes. The supernatant was collected in sterile container and kept at -20°C until used (Bearel, 1989).

(b) Tissues: oviduct, ovary, pouch shell gland, intestine and oesophagus was prepared as 20% suspension by pooling half gram of each tissue and adding the mixture to 10 ml Normal Saline in a mortar and grinding with sterile sand. The resulting homogenate was centrifuged at 1000 rpm for 5minuets. The supernatant was carefully removed and used for eggs inoculation (Versteeg, 1985).

2.7.2 Embryonated chicken eggs:

Seven day old embryonated chicken eggs were kindly obtained from the National Fowl Scheme and Corals farms. Eggs were incubated at 37°C in an eggs incubator. The eggs were used for virus isolation.

2.7.3 Inoculation:

For virus isolation five, 10 day-old embryonated chicken eggs were used for inoculation of each samples and one embryonated chicken egg was kept as control. Before inoculation eggs were candled to confirm the viability of embryos and to choose an area for inoculation which must be free of large blood vessels. The eggs were washed with sterile distilled water and then disinfected by swabbing with 70% alcohol.

A small hole was then made by a blunt needle in the labeled area between the air sac and the chorio-allantoic membrane and 0.2ml of the prepared inoculums was injected into allantoic cavity by using 1ml sterile syring. The inoculated eggs were rotated gently to ensure even distribution of the inoculums. Then the hole was sealed with molted wax and eggs were incubated at 37°C and candled daily. Embryos died after 24 hours incubation was discarded. The dead embryos were chilled for at least four an hours at 4°C before harvesting the allantoicy fluid (Beard, 1989).

2.7.4 Collection of Allantoic fluids and chorio allantoic membrane:

(a) Allantoic and amniotic fluids: The shell over the air sac was disinfected with 70% alcohol and removed with sterile forceps. The chorio allantoic membranes and the yolk sac membrane were gently ruptured using sterile forceps, forming a pool of fluids, which was aspirated with sterile syringe. Then the fluid was centrifuged at 1000 rpm for 5 minutes. The clarified fluid was kept at-20°C until used.

(b)The chorio allantoic membrane (CAM): After discarding the contents of the egg, the CAM was removed by sterile forceps and transferred into a Petri dish containing sterile normal saline. The CAM was examined for the presence of hemorrhage or thickness, which were suggestive for virus replication, then collected in sterile container and kept at -20°C.

2.7.5 Passage of isolates:

The harvested allantoic fluid was centrifuged at 1000 rpm for 5minutes. The supernatant 0.2ml containing antibiotics mixture was used for inoculation of another 5 embroyanated chicken eggs as described above. Three passages were made before the presence of the virus was detected by means of HA test.

2.8 Identification of virus:

This was based on.

(a) Death of embryos:

The eggs were examined by candling to detect embryos mortality. Embryos usually died 36 to 96 hours after inoculation (Beard, 1989).

(b) Hemagglutination test using chickens R BCs.

CHAPTER THREE

3. THE RESULT

3.1 Field observations:

3.1.1 Study area:

In this investigation, infected farms in three different areas in Khartoum state were visted. In Khartoum area, eighit farms were examined and total of 350 serum samples were collected. In Khartoum North area 16 farms were examined and total of 520 serum samples were collected. While in Omdurman area, three farms were examined and total of 130 serum samples were collected (Table1).

3.1.2 Birds breeds and production system:

The affected birds were of different breed. The breeds raised in these farms include hisex-white –brown, bovans and local breeds. All birds in these farms were raised for egg production. The system of management was open system.

3.1.3 Flock size:

Flock size varied between 1,000-5,000 as shown in table (1)

3.1.4 Clinical and post mortem observations:

In the investigated farms all birds looked apparently healthy. The main signs observed were drop in eggs production at peak of production, the eggs were thin-shelled, small in size and loss of shell pigment in brown eggs. Postmortem examination, the main lesions seen were edema of the uterine folds and presence of exudates in the pouch shell gland.

3.2 Serological tests:

Two serological tests, Hemagglutination and Elisa were used to examine serum sample collected.

3.2.1 Hemagglutination inhibition (HI) test:

When 1000 serum samples collected in this study were examined by (HI) test, 317 samples (31.7%) were found positive. The highest positive HI titre range (7-8 $\log_2$) were detected in samples collected from Khartoum North. The samples showed positive HI reactions in were 223 (45.88%), in Khartoum area (45.88%), in Khartoum area were 94 (29.38%), while no antibodies were detected by HI test in all serum samples collected from Omdurman area.

Table (1): Hemagglutination inhibition positive serum samples collected from three different areas in Khartoum state.

Samples source	Number of samples examined	Number of positive samples percent
Khartoum area		
Gebel Awellia (3)	120	41(34.17%)
Soba (1)	69	28(40.58%)
Elgreaf (2)	61	25(40.98%)
Buri (2)	100	Zero (0.0%)
Khartoum Total	350	94(29.38%)
Khartoum North area		
El kabashi (2)	60	21(35%)
El Zakiab (1)	96	50(72.46%)
El Sagai (2)	100	69(69%)
El Halefaia (2)	88	34(38.64%)
Kuku (1)	30	10(33.33%)
Shambat (9)	146	39(26.71%)
Khartoum North Total	520	223(45.88%)
Omdurman area		
Dar Alsalam	40	Zero (0.0%)
Al Sarha	50	Zero (0.0%)
Cero	40	Zero (0.0%)
Omdurman Total	130	Zero (0.0%)

Table (2): Hemagglutination inhibition titre expressed as log_2 of chicken serum collected from three different areas in Khartoum state.

Location	Number of samples examined	H1 titre (log_2)							
		$log_{2>1}$	2	3	4	5	6	7	8
Khartoum area									
Gebal Awellia	120	56	9	2	6	13	9	15	10
El Greaf (2)	061	5	0	0	2	8	5	20	21
Soba (1)	069	13	2	6	6	6	9	13	15
Buri (2)	100	-	-	-	-	-	-	-	-
Khartoum Total	350	162	11	8	14	27	22	48	46
Khartoum North area									
El Kabashi (2)	060	4	1	3	6	17	8	17	4
El Sagai (2)	100	44	0	0	2	4	1	26	23
ElHalifaia (2)	89	15	0	2	8	12	18	18	16
El Zakiab (1)	96	40	5	1	0	5	12	16	25
Kuku (1)	30	17	1	0	0	0	2	5	5
Shambat (8)	146	24	5	4	12	10	25	15	53
Khartoum North Total	520	134	12	10	28	48	60	97	126
Omdurman area									
Daralsalam	40	-	-	-	-	-	-	-	-
Alsarha	50	-	-	-	-	-	-	-	-
Cero	40	-	-	-	-	-	-	-	-
Omdurman Total	130	-	-	-	-	-	-	-	-

5.2.2 ELISA:

Two hundred Seventy eight serum samples were examined by ELISA test. The serological examination of serum samples collected from Khartoum, Khartoum North and Omdurman by ELISA test detected 14.2% positive samples. The highest number of positive samples was detected in Khartoum North area was16(9.34%) while the number of positive samples in Khartoum area was 13(4.68%). No positive reactor were detected by ELISA test among serum samples collected from Omdurman area as shown in table (3).

The highest titres (>400) were detected in two samples in Khartoum area (Elgreaf) and in 7 samples in Khartoum North area (Shambat).

Table (3): ELISA positive sera collected from three different areas in Khartoum state:

Locality	Number of samples examined	Number of positive samples (percent)
Khartoum area		
Gebel Awellia (3)	30	7 (23.33%)
Soba (1)	10	1 (10%)
Elgreaf (2)	20	5 (25%)
Buri (2)	20	0(0.0%)
Khartoum Total	80	13(16.25%)
Khartoum North area		
El Kabashi (2)	20	3 (15%)
Kuku (1)	10	2 (20%)
Elzakiab (1)	24	2 (8.33%)
Elsacai (2)	12	1 (8.33%)
Elhalifaia (2)	12	1 (8.33%)
Shambat (8)	84	17 (20.24%)
Khartoum total	162	26 (16.05%)
Omdurman area		
Dar Alsalam	12	0 (0.0%)
Al sarha	12	0 (0.0%)
Cero	12	0 (0.0%)
Omdurman Total	36	0 (0.0%)
Total	**278**	**39**

Table (3): ELISA titre of chickens sera collected from three different areas in Khartoum state

Locality	Number of samples examined	ELISA titre range Low (%)	Medium (%)	High(percent)
Khartoum area				
Gebe Awellia(3)	30	2 (6.87%)	5 (16.67%)	-
Soba (1)	10	1 (10%)	-	-
Elgreaf (2)	20	2 (10%)	1 (5%)	2 (10%)
Buri (2)	20	-	-	-
Khartoum total	80	5 (6.25%)	6 (7.5%)	2 (2.5%)
Khartoum North area				
El Kabashi (2)	20	1(5%)	2 (105)	-
Kuku (1)	10	1 (10%)	1 (10%)	-
Elsagai (2)	12	-	1 (8.33%)	-
Elzakiab (1)	24	1 (4.17)	1 (4.17%)	-
Elhalifaia (2)	12	-	1 (8.33%)	-
Shambat (8)	84	5 (5.95%)	5 (5.95%)	7 (8.335)
Khartoum North total	162	8 (4.94%)	11 (6.8%)	7 (4.32%)
Omdurman area				
Dar alsalam	12	-	-	-
Alsarha	12	-	-	-
Cero	12	-	-	-
Omdurman total	36	-	-	-
Total	**278**	**13**	**17**	**9**

-138 Low range

- 238 Medium rang

- 400 High range

3.3. Virus isolation:

Attempts were made to isolate EDS. 76 adenovirus, in embryonated chicken embryo. Three blind passages were made in embryonated chicken eggs. Allantoic fluid was checked after each passage for presence of HA to avian erythrocytes (0.8%). However, no HA was detected.

Table (5): Virus isolation: when passages were made in embryonated chicken eggs.

Type of samples	Number of examined samples	Number of positive sample (percent)
Oviduct – ovaries pouch shell gland	15	None (0.0%)
Intestine – oesophingus	15	None (0.0%)
Cloacal swabs	15	None (0.0%)
Total	**45**	**None (0.0%)**

CHAPTER FOUR

4. DISCUSSION

The present study was carried out to investigate the presence of Egg drop syndrome (EDS76) in Khartoum State. Khartoum State has the largest chicken population and greatest number of poultry farms in the Sudan. A severe drop in egg production accompanied by egg abnormalities, decreased shell thickness and size were observed in different breeds (Hisex-white–brown, Bovans and local chickens) at Khartoum State. A reduction in egg production varied from 30% to 45% was recorded. Mineral deposition on the shell surface of some eggs was noticed but shell- less eggs were not recorded. These observations were in consistence with that of Mc Feran *et al.* (1978), VanEck *et al.* 1979), Yamguchi *et al.* (1980), Firth *et al.* (1981), Bragg *et al.* (1991) and Bishop and Corrdozo (1986), who recorded signs of egg abnormalities consisting of lack of shell pigment, decreased shell thickness leading to cracks, decrease size and soft shelled or shell less eggs with either a fall in egg production or a failure to achieve predicted production levels. EDS 76 viruses (adenovirus) which agglutinate fowl erythrocytes have been isolated from this infected chickens (Calneck *et al.* 1991; Bragg *et al.* 1991).

In this investigation a total of 1000 serum samples collected from different breeds and different localities in Khartoum state and were examined for adenovirus (EDS.76) antibodies by HI test and ELISA test. Results of HI test detected EDS.76 virus antibodies and high level of HI titre of 8 log_2 was recorded in Shambat farms (Khartoum North area) and El Greaf (Khartoum area). Since there is no-vaccination program was adopted against an adenovirus (EDS-76) in Sudan, the presence of EDS-76 virus antibodies in the serum samples collected from the different breeds indicated previous adenovirus infection Ballal *et al.* (1994) also reported the presence of antibodies against EDS. 76 in different area in Khartoum State by HI test. World wide Mcferran (1977) reported the presence of antibodies against EDS 76 virus in northern Ireland. Bragg *et al.* (1991), Bisho and Gardosa (1996) also reported the presence of antibodies in laying hens serum in South Africa and Bolivia by HI test. Zhu *et al*. (1999) and Raj *et al.* (2003) reported the presence of antibodies against EDS 76 virus in chicken sera in India by ELISA test. It was found ELISA was efficacious in quantification of both vaccinal .and infection antibodies and could routinely be used for screening large numbers of field sera. This study reported for the first the use of ELISA test for detection of antibodies against EDS-76virus in the Sudan. ELISA was found to have 9.3.6% sensitivity and 98.7% specificity relative to haemagglutination inhibition test and the correlation coefficient for ELISA and HI titers was 0.793 (Raj *et al.,* 2004). However, the HI remain the test of choice for diagnosis of EDS-76 (David *et al.;* 1998; Raj *et al.,* 2004).

When there is series of small episodes of EDS-76, and if care was not taken to select samples throughout the house false negative results may be obtained by ELISA (Calnek *et al.,*1991). This may explain low detection rate (14.2%) by ELISA test when compared with detection rate (32.7%) by HI test observed in this study.

HI test and ELISA technique detected the presence of EDS.76 antibodies in sera of chicken collected from Khartoum and Khartoum North area, this confirmed the findings obtained by HI test. This study reveled, EDS.76 infection is more prevalent in Khartoum North area when compared Khartoum area. This may be because Khartoum North farms were located close together and were crowdy. Also the movement of workers between farms may play role in spread of the disease in Khartoum North.

Reused traies may also play role in transmission of the disease. (Calneck *et al.* 1991)

In Omdurman area no antibodies against EDS 76 virus was detected by HI or ELISA tests. This possible, because the farms there were very far away from each other and the farms were not crowdy. Calneck *et al.* (1991) reported that the crowdness enhance the horizontal transmission of the disease within the same poultry house.

However, epidemiological evidence suggests the virus is egg transmitted (Mc Ferran 1976). Hence, it is possible, the EDS–76 infected flocks in

Khartoum and Khartoum North areas were purchased from layers breeding units infected with EDS.76 virus and hence, the infection of these flocks was egg transmitted. It is also possible the infection has been carried by chickens from farm to a farm and then spread horizontally to other farm (Badstive and Smidt, 1978).

Three blind passages were made in embryonated chicken eggs but the virus was not isolated, this possibly because embryonated chicken eggs is not suitable for EDs 76 virus isolation. Duck and goose embryonated eggs are suitable for EDs 76 virus isolation. The virus was isolated in duck embryos by Mc Ferran (1977) and Yamaguchi *et al*. (1981). Unfortunately the duck and goose embryonated eggs are not available in the country. Similar to this study, the disease was diagnosed based on clinical observations, serological detection of antibodies and history of no vaccination (Ballal and Kheir 1994; Bishop and Cardozo, 1996).

In conclusion the present work confirmed the presence of EDS. 76 in the Sudan, it is therefore recommended that further studies should be carried out on etiology and epidemiology of the disease because of its great economic importance

REFERENCE

Alexander, D. J. (1990). Egg drop syndrome In: poultry Disease Edit F.T.W. Jordan. Third edition.

Ballal, A. G. and Kheir, S. A. M. (1994). Serological studies on flocks showing depressed egg production in the Sudan. Sudan J. Vet Res, **13**: 67-71.

Barrow, and Feltham, (1993). Cowan and steel. Manual of identification of Medical Bacteria.

Baxendale, W. (1978). Egg drop syndrome 76.Vet Rec, **102**: 285-286.

Baxendale, W; Luttlicken, D.; Hein, R. and Mcpherson; I. (1980). Egg drop syndrome 76. Avian Pathol, **9**: 77-91.

Bcard, C. W. (1989). Egg drop syndrome. In: Manual of isolation and Identification of Avian Pathogens.

Bishop, S.C. and Cardoz, O, P. (1996). Egg drop syndrome 76 in Bolivia. Trop Anim. Health Prod., **28** (3): 199-206.

Calnek, B.W. John Barnes, H.; Beard, C. W.; Reid, W. M. and Yoder, H. W. (1991). Diseases of Poultry. 9thed. Iowa State University press Ames, Iowa U. S.A

Calnek, B.W.; John Barnes, H.; Beard, C.W.; Reid, W.M. and Yoder, H. W. (1997). Diseases of Poultry. 10th ed. Iowa States University press Ames, Iowa U.S.A.

Cook, J. K. A. and Darbyshire, J. H. (1980). Egg drop syndrome 76. Avian Pathol, **9**: 437-443.

Darbyshire, J.H. and Peters, R.W. (1980). Egg drop syndrome . Avian Pathol. **9**:277-290.

David, E. Swayne, charirm. John R; John, Glisson, W. Mark, J. Jackwzrd ames, E; Pearson . Willie, M. Reed. (1998). A laboratory Manual for the Isolation and Identification of Avian Pathogens. Fourth edition Rose printing, Tallahassee, Florida.

Dhinakar, R.G.; Ratnabapaba, S.; Mathes waran, K.; Nachimuthu, K. (2004). Comparison of hemagglutination. inhibition test and ELISA in quantification of antibodies to egg drop syndrome virus. Acta Virol., **48**(3): 183-187.

Dhinakar, R.; Siva, G.; Kumar, S.; Mathes Waran, K.; Chandrasekhar, M.; Thiagarajan, V.; Nachinuthu, K. (2003). Detection of egg drop syndrome virus antigen or genome by enzyme-linked immuno sorbent assay or polymerase chain reaction. Avian Pathal., **32** (5): 545-550.

Fenner, F.; Petar, A.; Bachmann, E.; Paul, J.; Gibbs, Fredrich ,A.; Murphy, Michael, J. Studdert and David O white. (1987). Veterinary Virology. Academic press INC., London NW 17 DX, England.

Firth, G. A.; Hall, M. J. and McFerran, J. B. (1981). Egg drop syndrome. Aust. Vet. J., **57**:239.242.

Grist, N.R. (1979). Diagnostic Method. In: Clinical Virology. Third edition.Jordan, F.T.W. (1979). Poultry diseases. Bailliare Tind all, London Nw 17Dx, England.

Knipe, D. M.; Howley, P.M.; Griffcn, D.E.; Martion, M.A.; Lamb, R.A.; Roizman, Band straus, S.E. (2001). Adenovividae. In: Field. Virology Lippino Williams pp.2265-2291.

Mcferran, J. B.; McCracken, R.M.; Mckillop, E. R.; McNulty, M.S. and Collins, D. S. (1978b). Egg dropsy-syndrome. Avian Pathol; **7**: 35-47.

McFerran, J.B. (1997). Diseases of Poultry, 10th ed Edited by B.W Calnek, H.Johon Bames, C.W Beard, L. R.; McDouglad, Y. Msai.EditionBoard for the Americom Association of Avian Pathologist.

Merchant, I.A. and Packer, R.A. (1976). Veterinary Bacteriology and Virology. Seventh edition. Ames Iowa, U.S.A. the Iowa State University press.

Meulemans, G.; Dekegel, D.; Peters, J.P; van Meirhaeyhes, E. and Halen, P. (1979). Egg dropey-ndrome76. vlaams Dierge neesk d Tijd schr, 2:157.

Murphy, F. A.; Gibss, E.P.J.; Horzinek, M. C.; Studdert, M. J. (1999). Adenovividae. In: Veterinary Virology 3rded. Academic press. pp. 327-334.

OIE (1992) Egg drop Syndrome. 123-129.

VanEck, J.H.H.; Davelear, F.G.; Vanden, Heuvel-plesman, T.A.M.; Van Kol, V.; Kouwenhoven B.; and Guldie, F. H. (1976). Egg drop syndrome 76. Avian Pathol., **5**:261-272.

VanEck, J.H.H.; Lenbass, L.E.; Wensvoort, P., and Kouwenh, B. (1978). Egg drop syndrome76. Avian Pathol., 7:279-287.

Versteeg, J. (1985). A colour Atlas of Virology.

Yamaguchi, S.; Imada, T.; Kawamura, H.; Kawak ,M.I. (1981). Egg drop syndrome76. Avian Dis **25**:64-649.

Zack, L. and Kisary, J. (1984). Grouping of fowl adenoviruses based upon the restriction pattern of DNA generated by BAM Hi and Hind 111- Inter Virology, **22**:110-114.

Zhu, L; Jin, Q.; Zeng. L. (1999). Detection of egg drop syndrome virus antigen or genome by enzyme-linked immuno sorbent assay or polymerase chain reaction {Artide in Chinese}. Zhonghua shi.